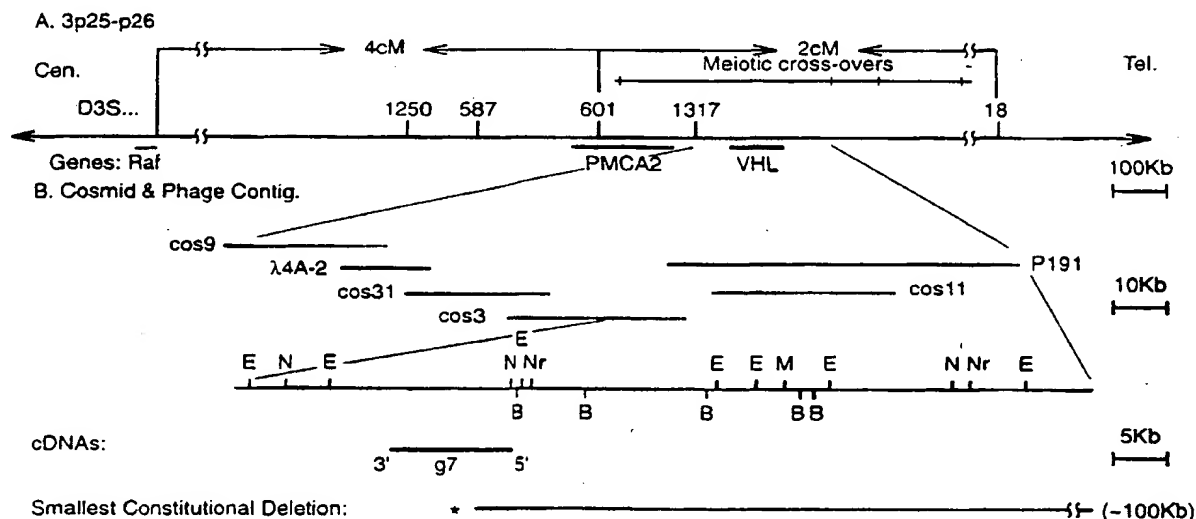




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(54) Title: VON HIPPEL-LINDAU (VHL) DISEASE GENE AND CORRESPONDING cDNA AND METHODS FOR DETECTING CARRIERS OF THE VHL DISEASE GENE



(57) Abstract

The invention is the Von Hippel-Lindau (VHL) disease gene and its corresponding cDNA. Methods for detecting carriers of the VHL disease gene using probes derived from the cDNAs are described.

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Title of the Invention

VON HIPPEL-LINDAU (VHL) DISEASE GENE AND
CORRESPONDING cDNA AND METHODS FOR DETECTING CARRIERS OF
THE VHL DISEASE GENE

Field of Invention

The invention is in the field of tumor suppressor genes. More specifically, the invention relates to the Von Hippel-Lindau (VHL) disease gene and its corresponding cDNA and to methods for detecting carriers of the VHL disease gene using probes derived from the cDNA.

Background of Invention

Von Hippel-Lindau (VHL) disease is a familial cancer syndrome. This disease is an autosomal dominant disorder and patients who are heterozygous for mutations in the VHL disease gene are predisposed to a variety of cancers, the most frequent being hemangioblastomas of the central nervous system and retina, renal cell carcinoma (RCC) and pheochromocytoma. The multisystem character of the illness, combined with the fact multiple tumors may form in each target organ, produces considerable morbidity and mortality as evidenced by the reduction in life expectancy of affected individuals to 49 years (McKusick, V.A., Mendelian Inheritance in Man (1983) Johns Hopkins University Press, Baltimore and London, p 534-535). Although the prevalence of VHL disease is only 1 in 36,000, because of its late onset most individuals have children before they realize they have inherited VHL disease. For many years, the only method of presymptomatic or prenatal diagnosis of the disease has been periodic examination of the eye, brain, and abdomen in all asymptomatic members of VHL families. Unfortunately, examination of all target organs is required to ensure detection of disease that may be limited to a single organ. In addition to the obvious

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inconvenience and the cost of these examinations, they have the additional drawback that they may not yield definitive diagnostic information. Therefore, in order to develop a method which allows the unequivocal diagnosis of VHL disease in individuals at risk, researchers have focused intensive efforts on identifying and isolating the VHL disease gene.

Results of this research have shown that the VHL disease gene is a member of the family of tumor suppressor genes (Tory, K. et al. J. Natl. Canc. Inst. (1989) 81:1097-1101; Maher, E.R. et al. J. Med. Genet. (1990) 27:311-314) and that it behaves in accordance with Knudson's theory of human carcinogenesis (Knudson, A., Proc. Natl. Acad. Sci. USA (1971) 68:816-823). In addition, the identification of DNA markers tightly linked to the VHL disease gene has allowed localization of the VHL disease gene to human chromosome 3p25-p26. (Hosoe, S. et al. Genomics (1990) 8:634-640; Maher, E.R. et al. Genomics (1990) 8:957-960; Glenn, G.M. et al. Hum. Genet. (1990) 87: 207-210, Latif, F. et al. Am J. Hum. Genet. (1992) 51 (suppl.) A63; Tory, K. et al. Genomics (1992) 13:275-286; Richards, F.M. et al. J. Med. Genet. (1993) 30:104-107); Seizinger, B.R. et al. Nature (1988) 332:268-269; Seizinger, B.R. et al. Proc. Natl. Acad. Sci. USA (1991) 88:2864-2868 and Vance J.M. et al. Am J. Hum. Genet. (1993) 51:203-209)). Recently, Glenn et al. (Glenn, G.M. et al. JAMA (1992) 1226-1231) have used DNA markers flanking the VHL disease gene as probes to detect linkage to the VHL disease gene via restriction fragment polymorphism analysis of DNA isolated from individuals who are members of families at risk for VHL disease. Although this DNA polymorphism method results in enhanced accuracy of identification of carriers of VHL disease gene, the method is inherently flawed in that DNA polymorphism analysis does not detect the VHL disease gene itself.

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More recently, a gene located in the VHL region has been cloned (Latif, F. et al. Cancer Res. (1993) 63:861-867). However, this gene was found to detect no mutations in VHL patients and thus, there are currently no available
5 methods which can identify carriers of the VHL disease gene with 100% accuracy. However, the recent identification and isolation of the VHL disease gene (Latif et al., Science, in press, "Identification of the von Hippel-Lindau Disease Tumor Suppressor Gene") and its
10 corresponding cDNA should allow the development of diagnostic methods which provide unequivocal detection of carriers of the VHL disease gene.

Summary of Invention

The present invention relates to the von Hippel-Lindau (VHL) disease gene and its corresponding cDNA.
15

The invention further relates to methods for detecting carriers of the VHL disease gene. The first method comprises analyzing DNA of a subject for mutations of the VHL disease gene associated with VHL disease.

20 The second method comprises analyzing RNA of a subject for mutations or alterations in the VHL-specific mRNA associated with VHL disease.

The third method comprises analyzing protein of a subject for alterations in VHL protein expression
25 associated with VHL disease.

The invention also encompasses recombinant VHL proteins derived from the VHL cDNA and antibodies directed against said VHL proteins or peptides derived therefrom.

The invention further relates to a method for
30 treating a carrier of VHL disease gene in which an expression vector containing a nucleic acid sequence representing wild-type VHL gene is administered to the carrier.

The invention also provides a diagnostic kit for
35 detecting carriers of the VHL disease gene. The kit

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comprises purified and isolated nucleic acid sequences useful as PCR primers in analyzing DNA or RNA for mutations of the VHL disease gene associated with VHL disease.

FIGURE LEGENDS

Figure 1 (Panel A) shows a genetic and physical map of the chromosome 3p region encompassing the VHL gene. Genetic and physical distances between selected markers are shown in centiMorgans and kilobases respectively. The location of selected cross-overs is indicated by crosses. Figure 1 (Panel B) shows the 160 kb cosmid and phage contig covering the VHL region. An enlarged restriction map of cos3, cos11, and phage p191 detailing the position of g7 cDNA isolated by screening a λ gt11 teratocarcinoma cDNA library with a conserved 7kb fragment from the centromeric end of cos11. The beginning of the smallest constitutional deletion is indicated by an asterisk and line. Restriction sites: B, Bam HI; E, Eco RI; N, Not I; Nr, Nru I; M, Mlu I.

Figures 2A and 2B show Northern blot analysis of the expression of the gene represented by g7 cDNA in various human tissues. Figure 2(A) shows low resolution blot containing 2 μ g poly A⁺ mRNA, the tissues are indicated above the lanes. Figure 2(B) shows a high resolution blot containing 1 μ g of poly A⁺ mRNA from: lane 1, fetal brain; lane 2, adult brain; lane 3, fetal kidney; lane 4, adult kidney; lane 5, cerebellum; lane 6, adult adrenal; and lane 7, prostate. The sizes of the transcripts were determined by the position of the 28S and 18S rRNA bands.

Figures 3A-3E show detection by Southern blotting analysis of rearrangement mutations in constitutional DNA of VHL affected patients using g7 cDNA as probe. (Figure 3A) DNA from lymphoblastoid cell lines of 7 unrelated VHL patients was digested with EcoRI and analyzed by standard blotting procedures. The normal

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invariant band is about 20 to 22 kb, the sizes of the aberrant bands probably resulting from intragenic deletions range from 4 to 25 kb. The patients code numbers are indicated above the lanes. (Figure 3B) DNAs from lymphoblastoid cell lines of pedigree members from a new mutation family (coded "S") digested with DraI, HindIII, and PstI. The pedigree with the position of the affected (dotted circles) and predicted (hatched circle) members is shown (Figure 3C). Males are represented by squares and females by circles. Genetic transmission of the mutant allele (the aberrant band) in a regular VHL family (coded "P"). The DNAs were digested with by EcoRI and analyzed by Southern blotting (Figure 3D); the pedigree is shown (Figure 3E).

Figure 4 shows Southern blot analysis of genomic DNA of VHL patients (only the initials of each patients name are given). The DNAs were digested with EcoRI and probed using different regions of g7 cDNA. Panel A: Total g7 cDNA probe; Panel B: 5' end probe, nucleotides 3-146; Panel C: 3' end probe nucleotides 1277-1600.

Figures 5A and 5B show the results of polymerase chain reaction-single stranded conformation analysis (PCR-SSCP) of the genomic DNA of VHL patients with the 8 bp insertion mutation (Table 1). Portions of the DNA sequencing gels are shown that display normal (Figure 5A) and 714insTTGTCCGT mutation sequences (Figure 5B). The DNA sequence is of the antisense strand; therefore, the inserted bases are 5'ACGGACAA3'. Adjacent to sequencing ladder are shown the positions of the insertion, and the nature of the insertion, as predicted from the sequence.

Figure 6 shows the results of a "zoo" blot illustrating evolutionary conservation of the putative VHL gene. The g7 cDNA shows cross species homology to DNA from mammals, birds, fly, and sea urchin. Lanes: 1, human (*Homo sapiens*); 2, chimpanzee (*Pan troglodytes*); 3, macaque (*Macaca fascicularis*); 4, cow (*Bovis domesticus*); 5, rat (*Rattus norvegicus*); 6, mouse (*Mus musculus*); 7,

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chicken (*Gallus domesticus*); 8, frog (*Xenopus laevis*); 9, fly (*Drosophila melanogaster*); 10, sea urchin (*Strongilocetrotus purpuratus*); and 11, yeast (*Saccharomyces ceriviseae*).

Detailed Description of the Invention

The present invention relates to the VHL disease gene and its corresponding cDNA. Recently, the region of human chromosome 3 containing the VHL disease gene has been cloned by genomic walking with yeast artificial chromosomes (YACS) and the cloned DNA recovered with cosmids from a chromosome 3 specific library (Latif et al. Science, in press). The phage 191 which contains the VHL disease gene was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 on May 13, 1993 (accession number 69311). This VHL disease gene represents the wild-type VHL gene where wild-type means the gene not causing VHL disease.

The present invention is also directed to a cDNA corresponding to the VHL disease gene. This cDNA sequence, designated g7, is set forth below as SEQ ID NO: 1 and was deposited with the American Type Culture Collection on May 13, 1993 (accession number 69312). This cDNA also has GenBank accession No. L15409.

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CCTCGCCTCC GTTACAACAG CCTACGGTGC TGGAGGATCC TTCTGCGCAC 50
GCGCACAGCC TCCGGCCGGC TATTTCCGCG AGCGCGTTCC ATCCTCTACC 100
GAGCGCGCGC GAAGACTACG GAGGTCTGACT CGGGAGCGCG CACGCAGCTC 150
CGCCCCGCGT CCGACCCGCG GATCCCGCGG CGTCCGGCCC GGGTGGTCTG 200
GATCGCGGAG GGAATGCCCC GGAGGGCGGA GAACTGGGAC GAGGCCGAGG 250
TAGGCGCGGA GGAGGCAGGC GTCGAAGAGT ACGGCCCTGA AGAAGACGGC 300
GGGGAGGAGT CGGGCGCCGA GGAGTCCGGC CCGGAAGAGT CCGGCCCGGA 350
GGAAGTGGGC GCCGAGGAGG AGATGGAGGC CGGGCGGCCG CGGCCCGTGC 400
TGCGCTCGGT GAACTCGCGC GAGCCCTCCC AGGTCATCTT CTGCAATCGC 450
AGTCCGCGCG TCGTGCTGCC CGTATGGCTC AACTTCGACG GCGAGCCGCA 500
GCCCTACCCA ACGCTGCCGC CTGGCACGGG CCGCCGCATC CACAGCTACC 550
GAGGTCACCT TTGGCTCTTC AGAGATGCAG GGACACACGA TGGGCTTCTG 600

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GTTAACCAAA CTGAATTATT TGTGCCATCT CTCAATGTTG ACGGACAGCC 650
 TATTTTTTGCC AATATCACAC TGCCAGTGTA TACTCTGAAA GAGCGATGCC 700
 TCCAGGTTGT CCGGAGCCTA GTCAAGCCTG AGAATTACAG GAGACTGGAC 750
 ATCGTCAGGT CGCTCTACGA AGATCTGGAA GACCACCCAA ATGTGCAGAA 800
 5 AGACCTGGAG CGGCTGACAC AGGAGCGCAT TGCACATCAA CGGATGGGAG 850
 ATTGAAGATT TCTGTTGAAA CTTACACTGT TTCATCTCAG CTTTTGATGG 900
 TACTGATGAG TCTTGATCTA GATACAGGAC TGGTTCCTTC CTTAGTTTCA 950
 AAGTGTCTCA TTCTCAGAGT AAAATAGGCA CCATTGCTTA AAAGAAAGTT1000
 AACTGACTTC ACTAGGCATT GTGATGTTTA GGGGCAAACA TCACAAAATG1050
 10 TAATTTAATG CCTGCCCATT AGAGAAGTAT TTATCAGGAG AAGGTGGTGG1100
 CATTTTTGCT TCCTAGTAAG TCAGGACAGC TTGTATGTAA GGAGGTTTAT1150
 ATAAGTAATT CAGTGGGAAT TGCAGCATAT CGTTTAATTT TAAGAAGGCA1200
 TTGGCATCTG CTTTTAATGG ATGTATAATA CATCCATTCT ACATCCGTAG1250
 CGGTTGGTGA CTTGTCTGCC TCCTGCTTTG GGAAGACTGA GGCATCCGTG1300
 15 AGGCAGGGAC AAGTCTTTCT CCTCTTTGAG ACCCCAGTGC CTGCACATCA1350
 TGAGCCTTCA GTCAGGGTTT CTCAGAGGAA CAAACCAGGG GACACTTTGT1400
 TAGAAAGTGC TTAGAGGTTC TGCCTCTATT TTTGTTGGGG GGTGGGAGAG1450
 GGGACCTTAA AATGTGTACA GTGAACAAAT GTCTTAAAGG GAATCATT1500
 TGTAGGAAGC ATTTTTTATA ATTTTCTAAG TCGTGCACTT TCTCGGTCCA1550
 20 CTCTTGTTGA AGTGCTGTTT TATTACTGTT TCTAAACTAG GATTGACATT1600
 CTACAGTTGT GATAATAGCA TTTTGTAAAC TTGCCATCCG CACAGAAAAT1650
 ACGAGAAAAT CTGCATGTTT GATTATAGTA TTAATGGACA AATAAGTTTT1700
 TGCTAAATGT GAGTATTTCT GTTCCTTTTT GTAAATATGT GACATTCCTG1750
 ATTGATTTGG GTTTTTTTGT TGTGTGTTT TTGTTTTGTT TTGTTTTTTT1800
 25 GGGATGGAGG GAATTC 1816

The abbreviations used for the nucleotides are those standardly used in the art.

The deduced amino acid sequence of the g7 cDNA is shown as SEQ ID NO:2 below and starts at nucleotide 1 of SEQ ID NO:1 and extends 851 nucleotides.

| | | | | | | | | | | | | | | | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 30 | Pro | Arg | Leu | Arg | Tyr | Asn | Ser | Leu | Arg | Cys | Trp | Arg | Ile | Leu | Leu |
| | | | | | 5 | | | | | 10 | | | | | 15 |
| | Arg | Thr | Arg | Thr | Ala | Ser | Gly | Arg | Leu | Phe | Pro | Arg | Ala | Arg | Ser |
| | | | | | 20 | | | | | 25 | | | | | 30 |
| | Ile | Leu | Tyr | Arg | Ala | Arg | Ala | Lys | Thr | Thr | Glu | Val | Asp | Ser | Gly |
| | | | | | 35 | | | | | 40 | | | | | 45 |
| 35 | Ala | Arg | Thr | Gln | Leu | Arg | Pro | Ala | Ser | Asp | Pro | Arg | Ile | Pro | Arg |
| | | | | | 50 | | | | | 55 | | | | | 60 |

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| | | | | | | | | | | | | | | | | | | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | Arg | Pro | Ala | Arg | Val | Val | Trp | Ile | Ala | Glu | Gly | Met | Pro | Arg | Arg | 65 | 70 | 75 |
| | Ala | Glu | Asn | Trp | Asp | Glu | Ala | Glu | Val | Gly | Ala | Glu | Glu | Ala | Gly | 80 | 85 | 90 |
| | Val | Glu | Glu | Tyr | Gly | Pro | Glu | Glu | Asp | Gly | Gly | Glu | Glu | Ser | Gly | 95 | 100 | 105 |
| 5 | Ala | Glu | Glu | Ser | Gly | Pro | Glu | Glu | Ser | Gly | Pro | Glu | Glu | Leu | Gly | 110 | 115 | 120 |
| | Ala | Glu | Glu | Glu | Met | Glu | Ala | Gly | Arg | Pro | Arg | Pro | Val | Leu | Arg | 125 | 130 | 135 |
| | Ser | Val | Asn | Ser | Arg | Glu | Pro | Ser | Gln | Val | Ile | Phe | Cys | Asn | Arg | 140 | 145 | 150 |
| | Ser | Pro | Arg | Val | Val | Leu | Pro | Val | Trp | Leu | Asn | Phe | Asp | Gly | Glu | 155 | 160 | 165 |
| 10 | Pro | Gln | Pro | Tyr | Pro | Thr | Leu | Pro | Pro | Gly | Thr | Gly | Arg | Arg | Ile | 170 | 175 | 180 |
| | His | Ser | Tyr | Arg | Gly | His | Leu | Trp | Leu | Phe | Arg | Asp | Ala | Gly | Thr | 185 | 190 | 195 |
| | His | Asp | Gly | Leu | Leu | Val | Asn | Gln | Thr | Glu | Leu | Phe | Val | Pro | Ser | 200 | 205 | 210 |
| | Leu | Asn | Val | Asp | Gly | Gln | Pro | Ile | Phe | Ala | Asn | Ile | Thr | Leu | Pro | 215 | 220 | 225 |
| 15 | Val | Tyr | Thr | Leu | Lys | Glu | Arg | Cys | Leu | Gln | Val | Val | Arg | Ser | Leu | 230 | 235 | 240 |
| | Val | Lys | Pro | Glu | Asn | Tyr | Arg | Arg | Leu | Asp | Ile | Val | Arg | Ser | Leu | 245 | 250 | 255 |
| | Tyr | Glu | Asp | Leu | Glu | Asp | His | Pro | Asn | Val | Gln | Lys | Asp | Leu | Glu | 260 | 265 | 270 |
| 20 | Arg | Leu | Thr | Gln | Glu | Arg | Ile | Ala | His | Gln | Arg | Met | Gly | Asp | | 275 | 280 | |

Variations are contemplated in the cDNA sequence shown in SEQ ID NO:1 which will result in a DNA sequence that is capable of directing production of analogs of the VHL protein shown in SEQ ID NO. 2. It should be noted that the DNA sequence set forth above represents a preferred embodiment of the present invention. Due to the degeneracy of the genetic code, it is to be understood that numerous choices of nucleotides may be made that will lead to a DNA sequence capable of directing production of the instant VHL protein or its analogs. As such, DNA sequences which are functionally equivalent to the sequence set forth above or which are functionally equivalent to sequences that would direct production of analogs of the VHL protein produced pursuant to the amino

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acid sequence set forth above, are intended to be encompassed within the present invention.

The term analog includes any protein or polypeptide having an amino acid residue sequence substantially identical to a sequence specifically shown herein in which one or more amino acid residues have been conservatively substituted with a functionally similar residue and which displays the functional aspects of the VHL protein as described herein. Examples of conservative substitutions include, for example, the substitution of one non-polar (i.e. hydrophobic) residue such as isoleucine, valine, leucine or methionine for another; the substitution of one polar (i.e. hydrophilic) residue for another, such as a substitution between arginine and lysine, between glutamine and asparagine, or between glycine and serine; the substitution of one basic residue such as lysine, arginine or histidine for another; or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The phrase conservative substitution may also include the use of a chemically derivatized residue in place of a non-derivatized residue provided that the resulting protein or polypeptide displays the requisite functional activity.

Chemical derivative refers to a VHL protein or polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Examples of such derivatized molecules include, but are not limited to, those molecules in which free amino groups have been derivatized to form, for example, amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters, or other types of esters or hydrazides. Free hydroxyl groups may be

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derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those proteins or peptides which contain one or more naturally-occurring amino acid derivatives of the twenty standard amino acids. For example, 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. A VHL protein or polypeptide of the present invention also includes any protein or polypeptide having one or more additions and/or deletions of residues relative to the sequence of a protein or polypeptide whose sequence is shown herein, so long as the requisite activity is maintained.

The present invention also relates to methods for detecting carriers of the VHL disease gene.

It is understood by one skilled in the art that the methods for detection disclosed in the present invention can be used prenatally to screen a fetus or presymptomatically to screen a subject at risk through his/her family history. In addition, these methods can be used to determine the involvement of the VHL disease gene in other human malignancies such as kidney, lung and bladder cancers.

In one embodiment of the invention, the method for detecting carriers of the VHL disease gene comprises analyzing the DNA of a subject for mutations of the VHL disease gene associated with VHL disease.

For purposes of the present invention, subject means a mammal and mutation means inversion, translocation, insertion, deletion or point mutation of the VHL disease gene.

For analysis of the DNA, a biological specimen

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is obtained from the subject. Examples of biological specimens that can be obtained for use in the present method include, but are not limited to, tissue biopsies, whole blood, urine, feces or other samples normally tested in the diagnosis of disease. Preferred biological specimens are whole blood or urine.

Although it is not always required, it is preferable to at least partially purify DNA from the biological specimen prior to analysis. For example, after disruption of cells in the specimen, nucleic acid can be extracted from contaminating cell debris and other protein substances by extraction of the sample with phenol. In phenol extraction, the aqueous sample is mixed with an approximately equal volume of redistilled phenol and centrifuged to separate the two phases. The aqueous phase containing the nucleic acid is removed and precipitated with ethanol to yield nucleic acid free of phenol. Alternatively, DNA can be purified from the biological sample according to Sidransky, D. et al. (Science (1992) 256:102-105; Science (1991) 252:706) or by the method of Glenn et al. (Glenn, G.M. et al. JAMA (1992) 267:1226-1231). The DNA to be analyzed can be either single- or double-stranded.

Methods for analyzing the DNA for mutations in the VHL disease gene include Southern blotting after digestion with the appropriate restriction enzymes (restriction fragment length polymorphism, RFLP) (Botstein, D. Amer. J. Hum. Genet. (1980) 69:201-205), denaturing gradient electrophoresis technique (Myers, R.M., Nature (1985) 313:495-498), oligonucleotide hybridization (Conner, R. et al., EMBO J. (1984) 3:13321-1326), RNase digestion of a duplex between a probe RNA and the target DNA (Winter, E. et al., Proc. Natl. Acad. Sci. U.S.A. (1985) 82:7575-7579), polymerase chain reaction (PCR) (Saiki, P.K. et al., Science (1988) 239:487-491;

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U.S. Patents 4,683,195 and 4,683,202), ligase chain reaction (LCR) (European Patent Application Nos. 0,320,308 and 0,439,182), and PCR-single stranded conformation analysis (PCR-SSCP) (Orita, M. et al., Genomics (1989) 5:874-879; Dean, M. et al. Cell (1990) 61:863-871). In one preferred embodiment, DNA is analyzed by Southern analysis.

The DNA to be analyzed via Southern analysis is digested with one or more restriction enzymes. The restriction enzymes to be used in the present invention are those enzymes for whom the presence or absence of their recognition site is linked to VHL disease. Preferred restriction enzyme include EcoRI, HindIII, PstI, DraI, BamHI, BglI, BglII, and PvuII. Following restriction digestion, resultant DNA fragments are separated by gel electrophoresis and the fragments are detected by hybridization with a labelled nucleic acid probe (Southern, E.M. J. Mol. Biol. (1975) 98:503-517).

The nucleic acid sequence used as a probe in Southern analysis can be labeled in single-stranded or double-stranded form. Labelling of the nucleic acid sequence can be carried out by techniques known to one skilled in the art. Such labelling techniques can include radiolabels and enzymes (Sambrook, J. et al. (1989) in "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Press, Plainview, New York). In addition, there are known non-radioactive techniques for signal amplification including methods for attaching chemical moieties to pyrimidine and purine rings (Dale, R.N.K. et al. (1973) Proc. Natl. Acad. Sci., 70:2238-2242; Heck, R.F. 1968) S. Am. Chem. Soc., 90:5518-5523), methods which allow detection by chemiluminescence (Barton, S.K. et al. (1992) J. Am. Chem. Soc., 114:8736-8740) and methods utilizing biotinylated nucleic acid probes (Johnson, T. K. et al. (1983) Anal. Biochem., 133:126-131; Erickson, P.F.

et al. (1982) J. of Immunology Methods, 51:241-249;
Matthaei, F.S. et al. (1986) Anal. Biochem., 157:123-128)
and methods which allow detection by fluorescence using
commercially available products. The size of the probe
5 can range from about 200 nucleotides to about several
kilobases. A preferred probe size is about 500 to about
2000 nucleotides. Each of the nucleic acid sequences used
as a probe in Southern analysis is substantially
homologous to the corresponding portion of the cDNA
10 sequence shown in SEQ ID NO:1. By "substantially
homologous" is meant a level of homology between the
nucleic acid sequence used as a probe and the
corresponding sequence shown in SEQ ID NO:1. Preferably,
the level of homology is in excess of 70%, most preferably
15 in excess of 80%, with a particularly preferred nucleic
acid sequence being in excess of 90% homologous with the
sequence shown in SEQ ID NO:1. Once the separated DNA
fragments are hybridized to the labelled nucleic acid
probes, the restriction digest pattern can be visualized
20 by autoradiography and examined for the presence or
absence of a restriction fragment length polymorphism
(RFLP) associated with VHL disease.

In a second preferred embodiment, the DNA is
analyzed for mutations in the VHL disease gene by PCR-SSCP
25 (Orita et al., (1989), Dean et al., (1990)). In this
method, each of the pairs of primers selected for use in
PCR are designed to hybridize with sequences in the VHL
disease gene which are an appropriate distance apart (at
least about 50 nucleotides) in the gene to permit
30 amplification and subsequent detection of mutations in the
amplification product. Primer pairs which can
specifically hybridize to such VHL gene sequences can be
derived from the VHL disease gene sequence. In a
preferred embodiment, the primers are derived from the
35 cDNA sequence shown in SEQ ID NO:1. Each primer of a pair

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is a single-stranded oligonucleotide of about 15 to about 50 bases in length which is complementary to a sequence at the 3' end of one of the strands of a double-stranded target sequence. Each pair comprises two such primers, one of which is complementary 3' end and the other of which is complementary to the other 3' end of the target sequence. The target sequence is generally about 100 to about 300 base pairs long but can be as large as 500-600 base pairs. Optimization of the amplification reaction to obtain sufficiently specific hybridization to the VHL disease gene is well within the skill in the art and is preferably achieved by adjusting the annealing temperature.

The present invention also provides purified and isolated pairs of primers for use in analysis of DNA for mutations in the VHL gene. The nucleic acid sequences of these primers is set forth below as SEQ ID NOS:3-8.

SEQ. ID. NO. 3

ATAGTGGAAA TACAGTAACG AGTTGGCCTA GCCTCGC

SEQ. ID. NO. 4

CCCAGCTGGG TCGGGCCTAA GCGCCGGGCC CGT

SEQ. ID. NO. 5

GTGGCTCTTT AACAACTTT GCTTGTCCTCG ATA

SEQ. ID. NO. 6

CAAGTGGTCT ATCCTGTACT TACCACAACA CCT

SEQ. ID. NO. 7

TGTATACTCT GAAAGAGCGA TGCCTCCAGG T

SEQ. ID. NO. 8

TACCATCAAA AGCTGAGATG AACAGTGTA AGT

where SEQ ID NO. 3 and SEQ ID NO. 4 represent one pair of primers; SEQ ID NO. 5 and SEQ ID NO. 6 represent a second pair of primers and SEQ ID NO. 7 and SEQ ID NO. 8 represent a third pair of primers.

The primers of this invention can be synthesized using any of the known methods of oligonucleotide

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synthesis (e.g., the phosphodiester method of Agarwal et al. 1972. Agnew. Chem. Int. Ed. Engl. 11:451, the phosphotriester method of Hsiung et al. 1979. Nucleic Acids Res. 6:1371, or the automated diethylphosphoramidite method of Beuacage et al. 1981. Tetrahedron Letters 22:1859-1862), or they can be isolated fragments of naturally occurring or cloned DNA. In addition, those skilled in the art would be aware that oligonucleotides can be synthesized by automated instruments sold by a variety of manufacturers or can be commercially custom ordered and prepared. In one embodiment, the primers can be derivatized to include a detectable label suitable for detecting and/or identifying the primer extension products (e.g., biotin, avidin, or radiolabeled dNTP's), or with a substance which aids in the isolation of the products of amplification (e.g. biotin or avidin). In a preferred embodiment, SEQ. ID. NO. 3 through SEQ. ID. NO. 8 are synthetic oligonucleotides.

In an alternative embodiment, primer pairs can be selected to hybridize to mutant forms of the VHL disease gene. The selected primer pairs will hybridize sufficiently specifically to the mutated gene sequences such that non-specific hybridization to wild-type VHL gene sequences will not prevent identification of the amplification product of the mutant gene sequence. Primer pairs which hybridize to mutations in the VHL gene sequence can be used to amplify specific mutant gene sequences present in the DNA of a biological sample.

The amplification products of PCR can be detected either directly or indirectly. In the PCR-SSCP method, direct detection of the amplification products is carried out via labelling of primer pairs. Labels suitable for labelling the primers of the present invention are known to one skilled in the art and include radioactive labels, biotin, avidin, enzymes and

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fluorescent molecules. The derived labels can be incorporated into the primers prior to performing the amplification reaction. A preferred labelling procedure utilizes radiolabeled ATP and T4 polynucleotide kinase (Sambrook, J. et al. (1989) in "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Press, Plainview, NY). Alternatively, the desired label can be incorporated into the primer extension products during the amplification reaction in the form of one or more labelled dNTPs. In the present invention, the labelled amplified PCR products can be analyzed for mutations of the VHL gene associated with VHL disease gene via separating the PCR products by denaturing polyacrylamide gel electrophoresis or via direct sequencing of the PCR-products.

In yet another embodiment, unlabelled amplification products can be analyzed for mutations in the VHL disease gene via hybridization with nucleic acid probes radioactively labelled or, labelled with biotin, in Southern blots or dot blots. Nucleic acid probes useful in the embodiment are those described earlier for Southern analysis.

In a second embodiment, the method for detecting carriers of the VHL disease gene comprises analyzing the RNA of a subject for mutations or alterations in VHL-specific mRNA associated with VHL disease.

For the analysis of RNA by this method, RNA derived from blood or a tumor biopsy sample is obtained from said subject where said tumors include, but are not limited to, tumors of the eye brain, liver, kidney, pancreas, and pheochromocytomas.

The RNA to be analyzed can be isolated from blood or tumor biopsy samples as whole cell RNA or as poly(A)⁺ RNA. Whole cell RNA can be isolated by methods known to those skilled in the art. Such methods include extraction of RNA by differential precipitation (Birnbiom,

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5 H.C. (1988) Nucleic Acids Res., 16:1487-1497), extraction of RNA by organic solvents (Chomczynski, P. et al. (1987) Anal. Biochem., 162:156-159) and extraction of RNA with strong denaturants (Chirgwin, J.M. et al. (1979) Biochemistry, 18:5294-5299). Poly(A)⁺ RNA can be selected from whole cell RNA by affinity chromatography on oligo-d(T) columns (Aviv, H. et al. (1972) Proc. Natl. Acad. Sci., 69:1408-1412). A preferred method of isolating RNA is extraction of whole cell RNA by acid-phenol (Chomczynski et al. 1987).

10 The methods for analyzing the RNA for alterations in the pattern or level of VHL specific mRNA expression linked to VHL disease include Northern blotting (Alwine, J.C. et al. (1977) Proc. Natl. Acad. Sci., 74:5350-5354); dot and slot hybridization (Kafatos, F.C. et al. (1979) Nucleic Acids Res., 7:1541-1522), filter hybridization (Hollander, M.C. et al. (1990) Biotechniques; 9:174-179), RNase protection (Sambrook, J. et al. (1989) in "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Press, Plainview, NY) and reverse-transcription polymerase chain reaction (RT-PCR) (Watson, J.D. et al. (1992) in "Recombinant DNA" Second Edition, W.H. Freeman and Company, New York). One preferred method is Northern blotting.

25 The nucleic acid sequence used as a probe for detecting VHL-specific mRNA expression is substantially homologous to SEQ. ID. NO. 1. By "substantially homologous" is meant a level of homology between the nucleic acid sequence and the cDNA sequence of SEQ ID NO.1. Preferably, the level of homology is in excess of 70% more preferably in excess on 80%, with a particularly preferred nucleic acid sequence being in excess of 90% homologous with the cDNA sequence shown in SEQ ID No. 1.

35 A most preferred method is reverse transcription-polymerase chain reaction (RT-PCR) where the

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primers used to amplify the cDNA produced via reverse transcription of RNA are derived from the cDNA sequence shown in SEQ ID No. 1. These primers can be labelled as described earlier and the RT-PCR products can be analyzed for mutations of the VHL gene associated with VHL disease via denaturing polyacrylamide gel electrophoresis of the RT-PCR products or via direct sequencing of the RT-PCR products.

The present invention also encompasses recombinant proteins derived from the cDNA shown in SEQ ID No. 1 and antibodies directed to said proteins (called VHL proteins). Recombinant VHL proteins can be produced by recombinant DNA methodology known to one skilled in the art. For example, a nucleic acid sequence capable of encoding a protein comprising all or part of the amino acid sequence shown in SEQ ID NO.2 can be cloned into a vector capable of being transferred into, and replicated in, a host organism. A suitable nucleic acid sequence for the purpose of this invention is the sequence shown in SEQ ID NO.1. Suitable expression vectors include, but are not limited to, vaccinia virus vectors include, baculovirus vectors, and Ecoli pTRCHIS (Invitrogen Co. San Diego). The recombinant expression vector produced by inserting a nucleic acid sequence capable of directing synthesis of VHL protein in a suitable expression vector can be transfected into E coli or into suitable eukaryotic cell systems by methods known to one skilled in the art.

Cells containing the expressed recombinant VHL protein, cell lysate from cells transfected with a recombinant expression vector or a culture supernatant containing the expressed VHL protein can be used as an immunogen to elicit production of anti-VHL antibodies in a mammal. Alternatively, one can generate synthetic peptides for use as immunogens from the amino acid sequence shown in SEQ ID NO 2. Preferred synthetic

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peptide sequences for use as an immunogen are shown below:

SEQ ID NO. 9:

Glu Glu Tyr Gly Pro Glu Glu Asp Gly Gly Glu Glu Ser Gly

SEQ ID NO. 10:

5 Gly Thr Gly Arg Arg Ile His Ser Tyr Arg Gly His Leu

While it is possible for the immunogen to be a administered to the mammal in pure or substantially pure form, it is preferable to present it as a pharmaceutical composition, formulation or preparation. Suitable mammals
10 for immunization include mice, rabbits and the like. The anti-VHL antibody of the present invention is typically produced by immunizing a mammal with an immunologically effective amount of synthetic peptide of this invention. The preparation of polyclonal or monoclonal antibodies
15 against such a peptide is well known in the art (Standt et al. (1988) J. Exp. Med. 157:687-704). The anti-VHL peptide antibody molecules induced by immunization of a mammal with the recombinant VHL protein are then collected from the mammal and those immunospecific for the VHL
20 protein are isolated to the extent desired by well known techniques such as, for example, immunochromatography.

In a third embodiment, the method for detecting carriers of the VHL disease gene comprises analyzing the protein of a subject for alterations in VHL protein
25 expression with VHL disease.

For analysis of protein by this method, protein is obtained from biological specimens such as tumor biopsy samples and urine and the like. The protein can be obtained as a crude lysate or it can be further purified
30 by methods known to one skilled in the art (Sambrook, J. et al. (1989) in "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor press, Plainview, NY).

Crude protein lysate can be analyzed for VHL protein by immunoassays using anti-VHL antibody.

35 Immunoassays of the present invention may be a

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radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, chemiluminescent assay, immunohistochemical assay and the like. Standard techniques known in the art for ELISA are described in
5 Method in Immunodiagnosis, 2nd Edition, Rose and Bigazzi, eds., John Wiley and Sons, 1980 and Campbell et al., Methods of Immunology, W.A. Benjamin, Inc., 1964, both of which are incorporated herein by reference. Such assays may be a direct, indirect, competitive, or noncompetitive
10 immunoassay as described in the art. (Oellerich, M. 1984. J. Clin. Chem. Clin. BioChem. 22:895-904).

Detection of the VHL protein anti-VHL antibody complex formed, can be accomplished by reaction of the complex with a secondary antibody such as labelled anti-
15 rabbit antibody. The label may be an enzyme which is detected by incubating the complex in the presence of a suitable fluorimetric or colorimetric reagent. Other detectable labels may also be used, such as radiolabels, or colloidal gold, and the like. The labelled VHL
20 protein-anti-VHL antibody complex is then visualized by autoradiography.

The present invention also relates to a method for treating a carrier of the VHL disease gene in which an expression vector containing a nucleic acid sequence
25 representing the wild type VHL gene is administered to the carrier. A nucleic acid sequence representing wild-type VHL gene is that shown in SEQ ID No. 1. Such nucleic acid sequence may be inserted into a suitable expression vector by methods known to those skilled in the art (Example 5).
30 Expression vectors suitable for producing high efficiency gene transfer in vivo include retroviral, adenoviral and vaccinia viral vectors.

Expression vectors containing a nucleic acid sequence representing wild-type VHL gene can be
35 administered intravenously, intramuscularly,

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subcutaneously, intraperitoneally or orally. A preferred route of administration is intravenously.

The invention also provides a diagnostic kit for detecting carriers of the VHL disease gene. This diagnostic kit comprises purified and isolated nucleic acid sequences according to SEQ ID. No. 3 through SEQ ID No. 8, said sequences useful as PCR primers in analyzing DNA for mutations of the VHL disease gene linked to VHL disease.

Any articles or patents referenced herein are incorporated by reference. The following examples illustrate various aspects of the invention but are in no way intended to limit the scope thereof.

Materials

The subjects analyzed in the following examples were kindred identified by ophthalmologists, urologists, medical geneticists and neurosurgeons in the United States, Europe, and Canada. The members of the families resided in Louisiana, Tennessee, Mississippi, Virginia, Pennsylvania, New York, Michigan, Quebec, Nova Scotia, United Kingdom, and the Netherlands. Medical records of each family member known to be affected were reviewed. Asymptomatic family members and family members in whom there was uncertainty about the diagnosis were examined after informed consent for occult evidence of the illness at the Clinical Center of the National Institutes of Health. The examination consisted of a history and physical examination of the scrotum. An asymptomatic member of a VHL family was considered to be affected if one or more of the following disease manifestations were detected: retinal angioma(s), spinal or cerebellar hemangioblastoma(s), pheochromocytoma(s), multiple pancreatic cysts, and multiple bilateral renal cysts accompanied by renal cell carcinoma. Disease diagnosis was made without knowledge of restriction fragment length

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polymorphism (RFLP) status.

Restriction enzymes were from Bethesda Research Laboratory (BRL) (Bethesda, MD), New England Biolabs (Beverly, MA) and Boehringer Mannheim (Indianapolis, IN) and were used as recommended by the manufacturers.

δ -³²PdCTP (~3000iu/mmol) was from Amersham (Arlington Heights, IL). The various human tissue polyadenylated RNAs used in Northern blotting were purchased from Clontech (Palo Alto, CA) as was the adult kidney double-stranded complementary DNA sample. PCR and RT-PCR kits were from Perkin Elmer/Cetus (Norwalk, CT); deoxynucleotide triphosphates and fluorescently labelled dideoxynucleotides were from Applied Biosystems, Inc. (Foster City, CA). Nylon membranes were purchased from MSI, Inc. (Westlore, MA).

Methods

Southern and Northern blottings, filter hybridization and probe labelling were by random priming were performed by standard protocols (Sambrook, J. et al. (1989)). DNA inserts were purified following the GeneClean (Bio 101) (BioRad, Richmond, CA) protocol and used for subcloning or labelling. Oligonucleotides used as primers in PCR or RT-PCR or for sequencing were synthesized on the Applied Biosystems, Inc. Model 392 DNA/RNA synthesizer, according to the manufacturers recommendations. Pulse field gel electrophoresis was carried out using CHEF-DR11 or CHEF mapper XA systems as described by the manufacturer (BioRad) under conditions optimal for obtaining the desired resolution.

The PCR was performed in a 50 ul reaction volume in a mixture containing 1uM of each primer, 250uM of each deoxynucleotide triphosphate, 5ul of 10X PCR buffer (500MM KCl; 120MM Tris-HCl, pH 8.0; 1.5MM MgCl₂; and 0.1% gelatin) and 1.25 units of AmpTaq (Cetus) DNA polymerase, in a first generation automated thermal cycler (Perkin-

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Elmer/Cetus). The PCR conditions consisted of 40 cycles of denaturation for one minute at 94°C, annealing for one minute at specified temperatures (55-65°C) and extension for 4 minutes at 72°C followed by 7 minutes of final extension of 72°C.

RNA Preparation and Northern Blotting - Total cellular RNA was isolated by extraction of lymphoblastoid cell lines of affected VHL patients or kidney tissues in guanidine thiocyanate followed by centrifugation through a 5.7 M CsCe cushion according to standard protocols (Sambrook, J. et al. (1989)). RNA samples were separated by electrophoresis in 1% agarose gels containing 2.2M formaldehyde, transferred to nylon membranes and hybridized to g7 cDNA probe (Sambrook, J. et al. (1989)).

RT-PCR - About 5 ug of total cellular RNA was isolated by extraction of lymphoblastoid cell lines or kidney tissues of VHL patients or 2.5 ng of normal adult kidney double-stranded complementary DNA samples were analyzed for expression using RT-PCR kit from Perkin-Elmer/Cetus. The primers were derived from the g7 cDNA sequence shown in SEQ ID NO. 1 and the reactions were run using various annealing temperatures. The reaction products were analyzed by gel electrophoresis and Southern blotting (Sambrook, J. et al (1989)).

EXAMPLES

Example 1

Isolation of the VHL Disease Gene.

The isolation of the VHL disease gene resulted from the use of positional cloning strategies (Latif et al., Cancer Res. (1993) 63:861-867; Trofatter et al., Cell (1993) 72:791-800 and The Huntington's Disease Collaborative Research Group; Cell (1993) 72:971-983) previously used in isolating disease genes and is described in Latif et al. (Science, in press, "Identification of the von Hippel-Lindau Disease Tumor

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Suppressor Gene"). Genetic and physical map of the chromosome 3p region encompassing the VHL gene is shown in Figure 1. The VHL locus was positioned on the map (Fig. 1, Panel A) by multipoint linkage analysis and meiotic mapping (Tory et al., 1989); the location of selected cross-overs is indicated by crosses.

YAC Library Screening and Analysis of YACs. Copies of the WU and CEPH YAC libraries were obtained from Dr. Craig Chinault (Baylor Institute of Human Genetics, Houston, Texas) and Dr. Daniel Cohen, respectively (centre d' Etude du Polymorphisme Humain, Paris). The WU and CEPH libraries are total human genomic DNA libraries constructed in the PYAC4 vector (Burke, D.T. et al. Science (1987) 236:806-812; Anand, R. et al. Nucleic Acids Res. (1990) 18: 1951-1956). These libraries were screened by sib selection using PCR-based techniques (Greene, E.D. et al., Proc. Natl. Acad Sci. (1990) 87:1213-1217) with primers for the D3S601, D3S587 and D3S18 loci in the VHL region (Figure 1). The sequences of the primers used to positively identify YACs Y52A10, YA101D4, Y132F2 and Y70D2 are shown below as SEQ ID No. 11 thru SEQ ID No. 16:

| <u>Locus/ Location</u> | <u>Designation</u> | <u>Sequence</u> |
|----------------------------|--------------------|----------------------------------|
| D3S18/3p26 | ML-1 | CACAAGTGAT GCCTTGTAGC TG No. 11 |
| D3S18/3p26 | ML-2 | CAGTAGTGTC CTGTATTTAG TG No. 12 |
| D3S601/3p25.3 | ML-7 | GTTGGCTATG GGTAGAATTG G No. 13 |
| D3S601/3p25.3 | ML-8 | CAGGGTAGCC TTGATCTAAG T No. 14 |
| D3S587/3p25.2 | ML-10 | GGAGGTCCTG AGAATATGTG TCC No. 15 |
| D3S587/3p25.2 | ML-11 | TGTTTCAGGCA CACAGTAGAT G No. 16 |

Screening Chromosome 3 Cosmid Library and Cosmid Contig Assembly. The chromosome 3 cosmid library was constructed as described in Lerman et al. (Lerman, M.I. et al. Hum. Genet. (1991) 86:567-577). This library was

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screened by colony hybridization (Sambrook, J. et al. (1989)) using the YAC DNA inserts as probes as described in Baxendale et al. (Baxendale, S. et al. Nucl. Acids Res. (1991) 19:6651). After labeling with ^{32}P -dCTP, the probes were preassociated with a 1000X excess of sheared human DNA. Cosmid contigs were constructed by finding overlapping bands on Southern blots of EcoRI-digested cosmids using whole cosmids as probes. Gaps in the cosmid contigs were closed by chromosome walking using insert-end fragment probes, which were identified by restriction mapping and hybridization to restricted genomic DNA. These insert-end fragment probes were used for each walk step. Figure 1 shows the 160 kb cosmid and phage contig covering the VHL region. The phage T42 was isolated by screening a total genomic phage library with YAC DNA inserts as described above. The phage p191, which contains the VHL disease gene, was isolated by screening a three-hit P1 phage genomic library (Genome System, Inc. St. Louis, MO) with primers chosen from within an exon of the g7 cDNA sequence shown in SEQ ID NO. 1. The phage p191 was deposited with the ATCC on May 13, 1993.

Example 2

Isolation of a cDNA Corresponding to VHL Disease Gene

Screening cDNA Libraries. A λ gt11 teratocarcinoma library (gift of Dr. Maxine Singer, National Cancer Institute) was screened by plaque hybridization (Sambrook, J. et al. (1989)) to 10^6 filter-immobilized cDNA phage clones at a density of 4×10^4 pfu/150-mm filter. Figure 1 (Panel B) shows the position of the g7 cDNA isolated by screening the λ gt11 teratocarcinoma cDNA library with a conserved 7 kb fragment at the centromeric end of cos11 used as a probe in the screening. The orientation of the g7 cDNA was established by sequencing and restriction mapping to the contig. The beginning of

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the smallest constitutional deletion is indicated by an asterisk and line. Restriction sites: B, Bam HI; E, Eco RI; N, Not I; Nr, Nru I; M, Mlu I.

cDNA Sequence and Sequence Analysis. The g7
5 cDNA clone was sub-cloned into the Bluescript KS (+)
plasmid (Stratagene, La Jolla, CA). Double-stranded
plasmid DNA was used in sequencing reactions performed
with Tag Dye Deoxy terminator cycle sequencing kits
(Applied Biosystems, Inc.). All sequences were obtained
10 by running the reactions in an ABI 373A automatic
sequencing system (Applied Biosystems, Inc.). Initial
sequencing was performed with T3 and T7 primers, and
"walking" primers were then constructed to continue
sequencing. The cDNA clone was sequenced multiple times
15 in one orientation or both orientations. Database
searching, sequence editing, sequence assembly, and
sequence analysis were carried out with the University of
Wisconsin Genetics Computer Group sequence analysis
software package, version 7.0 (Devereaux, J. et al. Nucl.
20 Acids Rev. (1984) 12:387-395). The sequence of the g7
cDNA is shown in SEQ ID No. 1. This cDNA was deposited
with the ATCC on May 13, 1993. The cDNA sequence revealed
an open reading frame (ORF) of 284 amino acids indicating
that the rest represents part of the 3' untranslated
25 region of the mRNA. This ORF showed a high probability
score (> 95%) for being a protein coding sequence Fickett,
J.W., Nucl. Acids Rev. (1982) 10:5303). Neither the
nucleotide nor the predicted amino acid sequences showed
any significant homology to genes or proteins in the
30 databases.

Example 3

Detection of g7-Specific mRNA Expression in Target Tissues

RNA Preparation and Northern Blotting Analysis.

To identify the VHL gene, we evaluated the g7 loci was
35 evaluated by analyzing its expression in target tissues.

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The expression pattern of the g7 gene was examined by Northern (RNA) blotting. Figure 2A shows a low resolution blot where each lane contains poly A⁺ mRNA (2 μ g) from: lane 1, fetal brain; lane 2, adult brain; lane 3, fetal kidney; lane 4, adult kidney; lane 5, adult cerebellum; lane 6, adult adrenal; and lane 7, adult prostate while Figure 2B shows a high resolution blot of 1 μ g of poly A⁺ mRNA from tissues as indicated in Figure 2A. The sizes of the transcripts were determined from the position of the 28S and 18S rRNA bands of total RNA run on the same gel. Transcripts were observed in all human tissues tested, including brain and kidney, tissues frequently affected in VHL disease. The transcripts were of two distinct sizes, 6 and 6.5 kb, and were expressed in a tissue-specific and developmentally selective manner, i.e. only 6 kb or the 6.5 kb species was expressed in fetal brain and fetal kidney, while both were expressed in adult tissues. The two transcripts may represent alternatively spliced forms of g7 mRNA.

Example 4

Detection of Mutations of the VHL Disease Gene Associated With VHL Disease

RT-PCR Studies of Gene Expression. In order to detect mutations in constitutional DNA of affected patients in pedigrees and in new mutation patients, was conducted an extensive search for mutations (i.e. small intragenic and nonoverlapping deletions or insertions) which were of the loss-of-function type was conducted in constitutional DNA derived from 221 unrelated VHL patients. Southern blot analysis of genomic DNA isolated from the blood (Sambrook, J. et al. (1989)) of seven patients and then digested with EcoRI is shown in Figure 3A. This blot was probed using the g7 cDNA as probe and this probe has been shown to detect a single invariant 20-22 kb EcoRI fragment in normal DNA, as determined by previous tests on more

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than 100 unrelated DNA samples provided by Centre d'Etude du Polymorphisme Humain (CEPH). A high incidence ($\geq 12\%$) of aberrant bands was observed with the bands ranging in size from 4 to 25 kb (Figure 3A) and thus classified these VHL patients were thus classified as new mutations.

In order to determine that the single aberrant bands originating from the 20-22 kb invariant fragment were deletions or insertions within this fragment or deletions removing the flanking Eco RI sites, Southern blot analysis was conducted with several other restriction enzyme digests besides Eco RI (Bam HI, Bgl I, Bgl II, Dra I, Eco RV, Hind III, Pst I, and Pvu II). The results of the Southern analysis with a few of these enzymes is shown in Figure 3B. These results demonstrated that the mutations were transmitted with the disease (Figure 3C). Figure 3D shows the results of Southern blotting analysis of DNA isolated from a regular VHL family (coded "P") and digested with EcoRI. The results clearly demonstrate transmission of the mutant allele (the aberrant band) in this VHL family (Figures 3D and 3E).

Example 5

Detection and Mapping of Deletions of the VHL Disease Gene

To prove the presence of deletions and to map them precisely, subfragments representing region of the g7 cDNA generated by PCR used as probes in Southern blotting analysis of genomic DNA isolated from blood of VHL patients and digested with EcoRI. (Figure 4, where the probes used in each panel are: Figure 4A, total g7 cDNA; Figure 4B, nucleotides 3-146 of g7 cDNA; and Figure 4C, nucleotides 1277-1600 of g7 cDNA). The results unequivocally demonstrated that 18 of the rearrangements were deletions as only part of the cDNA failed to detect the novel band in each patient (Figure 4).

These deletions could then be classified into three groups as shown in Table 1.

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TABLE 1

Deletion analysis of VHL patients with aberrant bands at the VHL locus (detected by g7 cDNA).

| Patient Code | Probe : cDNA 5'--->3' residue (s) | | | | | | Aberrant Band (kb) | Apparent Deletion Size (kb) |
|--------------|-----------------------------------|---------|---------|---------|----------|-----------|--------------------|-----------------------------|
| | 3-146 | 169-391 | 291-501 | 585-940 | 921-1231 | 1277-1600 | | |
| 3567 | ND | ND | ND | ND | ND | ND | 14 | ? |
| 3607 | ND | ND | ND | ND | ND | ND | 12 | ? |
| 3639 | ND | ND | ND | ND | ND | ND | 14 | ? |
| 3648 | ND | ND | ND | ND | ND | ND | 13 | ? |
| 3654 | ND | ND | ND | ND | ND | ND | 14 | ? |
| JD | ND | ND | ND | ND | ND | ND | 17 | ? |
| PEM | ND | ND | ND | ND | ND | ND | 15 | ? |
| MS | ND | ND | ND | ND | ND | ND | 15 | ? |
| KA | ND | ND | ND | ND | ND | ND | 15 | ? |
| 3547 | D | D | D | ND | ND | ND | 23-25 | 15-18 |
| JM | D | D | D | ND | ND | ND | 23-25 | 15-18 |
| GD | D | D | D | ND | ND | ND | 23-25 | 15-18 |
| 3512 | ND | ND | ND | ND | D | D | 10 | 11 |
| 3516 | ND | ND | ND | ND | D | D | 10 | 11 |
| 3557 | ND | ND | ND | ND | D | D | 10 | 11 |
| 3574 | ND | ND | ND | ND | D | D | 10 | 11 |
| VIA | ND | ND | ND | ND | D | D | 10 | 11 |
| IC | ND | ND | ND | ND | D | D | 10 | 11 |
| NE | ND | ND | ND | ND | D | D | 10 | 11 |
| EP | ND | ND | ND | ND | D | D | 10 | 11 |
| MO | ND | ND | ND | ND | D | D | 10 | 11 |
| 3569 | ND | ND | ND | D | D | D | 12 | 9 |
| 3667 | ND | ND | ND | D | D | D | 10 | 11 |
| 3761 | ND | ND | ND | D | D | D | 4 | 17 |
| 3819 | ND | ND | ND | D | D | D | 12 | 9 |

ND = Not deleted

D = Deleted

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The finding of three overlapping deletions within the same cDNA provides strong evidence for the identification of the g7 cDNA as the VHL gene.

Example 6

5 Detection of Intragenic Deletions or
 Insertions by PCR-SSCP and RT-PCR

To find intragenic deletions or insertions, genomic DNA isolated from VHL patient lymphoblastoid cell lines (Lymphoblastoid cells were immortalized by transformation with Epstein Barr Virus according to standard protocols (Nilison, K. et al., Adv. Cancer Res. (1982) 37:319-380)) was analyzed for alterations by PCR-single-strand-conformational polymorphism (PCR-SSCP) analysis using primers shown in SEQ ID NO. 3 thru SEQ ID NO. 8 and RNA isolated from sporadic renal cell carcinoma (RCC) cell lines (Anglard, P. et al. Cancer Res. (1992) 52:348-356) was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). The primers used for RT-PCR of the RCC cell lines are shown as SEQ ID NO. 17 thru SEQ ID NO. 20:

20 SEQ ID NO. 17

CATCTTCTGC AATCGCAGTC CGCGCGT

 SEQ ID NO. 18

CAAAAGCTGA GATGAAACAG TGTAAGT

25 SEQ ID NO. 19

GTTTGGTTAA CCAGAAGCCC ATCGT

 SEQ ID NO. 20

GATGGGCTTC TGGTTAACCA AACT

30 whose SEQ ID NO. 17 and NO. 18 are on pair of primers and
 SEQ ID NO. 19 and SEQ ID NO. 20 are a second pair. The
 results of these analyses are shown in Table 2.

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TABLE 2

Germ-line (VHL) and somatic (sporadic RCC) mutations in the VHL candidate gene.

| 5 | Patients | Mutation | Consequence |
|----|----------------------|---------------------------------------|---|
| | VHL family | | |
| | "VA" | 8 bp (TTGTCCGT) insertion after NT714 | frameshift |
| 10 | "E" | 9 bp in-frame deletion (NT456-464) | Three amino acid (153-154) deletion (Arg Val Val) |
| | "CS" | 3 bp in-frame deletion (NT434-436) | One amino acid deletion (146, Ile) |
| 15 | Sporadic RCC | | |
| | "UOK118" | 1 bp deletion (NT737) | frameshift |
| | "UMRC5" | 1 bp deletion (NT737) | frameshift |
| 20 | "UMRC6" | 10 bp deletion (NT715-724) | frameshift |
| | "A498" | 5 bp deletion (NT638-642) | frameshift |
| | "UOK151" | nonsense C → A (NT761) transversion | stop codon |
| 25 | *NT = nucleotide(s). | | |

30

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The finding of three overlapping deletions within the same cDNA provides strong evidence for the identification of the g7 cDNA as the VHL gene.

Example 6

5 Detection of Intragenic Deletions or Insertions by PCR-SSCP and RT-PCR

To find intragenic deletions or insertions, genomic DNA isolated from VHL patient lymphoblastoid cell lines (Lymphoblastoid cells were immortalized by
10 transformation with Epstein Barr Virus according to standard protocols (Nilison, K. et al., Adv. Cancer Res. (1982) 37:319-380)) was analyzed for alterations by PCR-single-strand-conformational polymorphism (PCR-SSCP) analysis using primers shown in SEQ ID NO. 3 thru SEQ ID
15 NO. 8 and RNA isolated from sporadic renal cell carcinoma (RCC) cell lines (Anglard, P. et al. Cancer Res. (1992) 52:348-356) was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). The primers used for RT-PCR of the RCC cell lines are shown as SEQ ID NO. 17
20 thru SEQ ID NO. 20:

SEQ ID NO. 17

CATCTTCTGC AATCGCAGTC CGCGCGT

SEQ ID NO. 18

CAAAAGCTGA GATGAAACAG TGTAAGT

25 SEQ ID NO. 19

GTTTGGTTAA CCAGAAGCCC ATCGT

SEQ ID NO. 20

GATGGGCTTC TGGTTAACCA AACT

whose SEQ ID NO. 17 and NO. 18 are on pair of primers and
30 SEQ ID NO. 19 and SEQ ID NO. 20 are a second pair. The results of these analyses are shown in Table 2.

- 33 -

leading to the removal of amino acid 146 (isoleucine).
Finally, patient "E" had an in-frame 9 bp deletion
(nucleotides 456 to 464) that resulted in the removal of
three amino acids (Arg Val Val) at position 153-155.

5 These combined results strongly support the conclusion
that the g7 gene represents the VHL and the sporadic RCC
tumor suppressor gene.

Example 7

Conservation of the g7 cDNA Across Species

10 In order to determine whether the g7 cDNA is
highly conserved across species ranging from mammals to
Drosophila and sea urchin, Zoo blotting using g7 cDNA as a
probe was performed on DNA isolated from human (*Homo*
15 *sapiens*), chimpanzee (*Pan troglodytes*), macaque (*Macaca*
fascicularis), cow (*Bovis domesticus*), rat (*Rattus*
norvigicus), mouse (*Mus musculus*), chicken (*Gallus*
domesticus), frog (*Xenopus laevis*), fly (*Drosophila*
melanogaster), sea urchin (*Strongilocetrotus purpuratus*),
and yeast (*Saccharomyces ceriviseae*), all purchased from
20 BIOS Laboratories (New Haven, CT, USA).

(Pre)Hybridization was done in Church buffer [G. M. Church
and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 1991
(1984)] at 65°C for 18 hours. Blots were washed in 0.1 x
Church buffer at 60°C for 60 min. The results of the zoo
25 blot are shown in Figure 6. The results demonstrate an
extensive evolutionary conservation which is indicative of
g7 serving a basic life function and also, of g7 having a
tumor suppressor role.

The contents of all citations, i.e., journal
30 articles, patents and the like, are incorporated herein by
reference.

It is understood that the examples and
embodiments described herein are for illustrative purposes
and that various modifications and changes in light
35 thereof to persons skilled in the art are included within

- 32 -

RCC were chosen because according to Knudson's dictum (Knudson (1971)) sporadic cancers should be associated with mutations in the same loci affected in the hereditary form of the same malignancy. So far aberrant patterns have been identified in five RCC cell lines and proved four of them have been proven to be small (1 to 10 bp) deletions creating frameshift mutations and truncated proteins (TABLE 2). The cell lines UMRC5 and RCC "UOK118" have the same 1 bp deletion at nucleotide 737, amino acid 246, creating 28 new amino acids followed by a stop codon. Incidentally, this deletion creates a new Eco RI site, leading to two aberrant bands on Southern blots (not shown). Line UMRC6 has a 10 bp deletion (nucleotides 715 to 724) creating a frameshift such that 32 new amino acids are present followed by a new stop codon. Finally, line A498 has a 5 bp deletion (nucleotides 638 to 642) leading to a premature stop after new 62 amino acids. In the fifth RCC cell line, UOK151, the change is a nonsense (stop codon) mutation resulting from a C to A transversion at nucleotide 761 (TCG → TAG), creating a truncated protein. These data suggest that the VHL disease gene plays an important role in sporadic kidney cancer. As such, RT-PCR or PCR-SSCP as described in this application can be used as diagnostic methods to distinguish primary kidney tumors from tumors that spread to the kidney from other tissues or organs and to distinguish different histological types of kidney tumors.

In the DNA of the VHL lymphoblastioid cell lines derived from VHL patients, SSCP aberrant patterns segregating with the disease were also detected using primers shown in SEQ ID NO. 3 thru SEQ ID NO. 8. One (patient "VA") was found to be an 8 bp (TTGTCCGT) insertion after nucleotide 714. This insertion created a shift in the reading frame and a truncated protein. The second patient ("CS") had an in-frame 3 bp deletions

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANTS: THE GOVERNMENT OF THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES
- (ii) TITLE OF INVENTION: VON HIPPEL-LINDAU (VHL) DISEASE GENE AND CORRESPONDING cDNA AND METHODS FOR DETECTING CARRIERS OF THE VHL DISEASE GENE
- 10 (iii) NUMBER OF SEQUENCES: 20
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: MORGAN & FINNEGAN
(B) STREET: 345 PARK AVENUE
(C) CITY: NEW YORK
(D) STATE: NEW YORK
(E) COUNTRY: USA
15 (F) ZIP: 10154
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: FLOPPY DISK
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WORDPERFECT 5.1
- 20 (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US94/_____
(B) FILING DATE: 12-MAY-1994
- (vii) PRIOR APPLICATION DATE:
(A) APPLICATION NUMBER: 08/061,889
(B) FILING DATE: 14-MAY-1993
25 (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: RICHARD W. BORK
(B) REGISTRATION NUMBER: 36,459
(C) REFERENCE/DOCKET NUMBER: 2026-4078PCT
- 30 (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (212) 758-4800
(B) TELEFAX: (212) 751-6849
(C) TELEX: 421792
- (2) INFORMATION FOR SEQ ID NO:1:
- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1816 base pairs
(B) TYPE: nucleic acid

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the spirit and purview of this application and scope of
the appended claims.

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- 37 -

TTCTCAGAGT AAAATAGGCA CCATTGCTTA AAAGAAAGTT 1000
AACTGACTTC ACTAGGCATT GTGATGTTTA GGGGCAAACA 1040
TCACAAAATG TAATTTAATG CCTGCCCATT AGAGAAGTAT 1080
5 TTATCAGGAG AAGGTGGTGG CATTTTTGCT TCCTAGTAAG 1120
TCAGGACAGC TTGTATGTAA GGAGGTTTAT ATAAGTAATT 1160
CAGTGGGAAT TGCAGCATAT CGTTTAATTT TAAGAAGGCA 1200
TTGGCATCTG CTTTTAATGG ATGTATAATA CATCCATTCT 1240
10 ACATCCGTAG CGGTGGTGA CTTGTCTGCC TCCTGCTTTG 1280
GGAAGACTGA GGCATCCGTG AGGCAGGGAC AAGTCTTTCT 1320
CCTCTTTGAG ACCCCAGTGC CTGCACATCA TGAGCCTTCA 1360
GTCAGGGTTT CTCAGAGGAA CAAACCAGGG GACACTTGT 1400
15 TAGAAAGTGC TTAGAGGTTC TGCCTCTATT TTTGTTGGGG 1440
GGTGGGAGAG GGGACCTTAA AATGTGTACA GTGAACAAAT 1480
GTCTTAAAGG GAATCATTTT TGTAGGAAGC ATTTTTTATA 1520
ATTTTCTAAG TCGTGCACTT TCTCGGTCCA CTCTTGTTGA 1560
20 AGTGCTGTTT TATTACTGTT TCTAAACTAG GATTGACATT 1600
CTACAGTTGT GATAATAGCA TTTTGTAAAC TTGCCATCCG 1640
CACAGAAAAT ACGAGAAAAT CTGCATGTTT GATTATAGTA 1680
25 TTAATGGACA AATAAGTTTT TGCTAAATGT GAGTATTTCT 1720
GTTCCTTTTT GTAAATATGT GACATTCCTG ATTGATTGG 1760
GTTTTTTTGT TGTGTGTGTT TTGTTTTGTT TTGTTTTTTT 1800
GGGATGGAGG GAATTC 1816

30

(2) INFORMATION FOR SEQ ID NO:2:

(i)

SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 284 amino acid residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

35

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(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

| | | |
|----|--|-----|
| 5 | CCTCGCCTCC GTTACAACAG CCTACGGTGC TGGAGGATCC | 40 |
| | TTCTGCGCAC GCGCACAGCC TCCGGCCGGC TATTTCCGCG | 80 |
| | AGCGCGTTCC ATCCTCTACC GAGCGCGCGC GAAGACTACG | 120 |
| | GAGGTCGACT CGGGAGCGCG CACGCAGCTC CGCCCCGCGT | 160 |
| 10 | CCGACCCGCG GATCCCGCGG CGTCCGGCCC GGGTGGTCTG | 200 |
| | GATCGCGGAG GGAATGCCCC GGAGGGCGGA GAACTGGGAC | 240 |
| | GAGGCCGAGG TAGGCGCGGA GGAGGCAGGC GTCGAAGAGT | 280 |
| | ACGGCCCTGA AGAAGACGGC GGGGAGGAGT CGGGCGCCGA | 320 |
| 15 | GGAGTCCGGC CCGGAAGAGT CCGGCCCGGA GGA ACTGGGC | 360 |
| | GCCGAGGAGG AGATGGAGGC CGGGCGGCCG CGGCCCGTGC | 400 |
| | TGCGCTCGGT GAACTCGCGC GAGCCCTCCC AGGTCATCTT | 440 |
| | CTGCAATCGC AGTCCGCGCG TCGTGCTGCC CGTATGGCTC | 480 |
| 20 | AACTTCGACG GCGAGCCGCA GCCCTACCCA ACGCTGCCGC | 520 |
| | CTGGCACGGG CCGCCGCATC CACAGCTACC GAGGTCACCT | 560 |
| | TTGGCTCTTC AGAGATGCAG GGACACACGA TGGGCTTCTG | 600 |
| 25 | GTTAACCAAA CTGAATTATT TGTGCCATCT CTCAATGTTG | 640 |
| | ACGGACAGCC TATTTTGTGCC AATATCACAC TGCCAGTGTA | 680 |
| | TACTCTGAAA GAGCGATGCC TCCAGGTTGT CCGGAGCCTA | 720 |
| | GTCAAGCCTG AGAATTACAG GAGACTGGAC ATCGTCAGGT | 760 |
| 30 | CGCTCTACGA AGATCTGGAA GACCACCCAA ATGTGCAGAA | 800 |
| | AGACCTGGAG CGGCTGACAC AGGAGCGCAT TGCACATCAA | 840 |
| | CGGATGGGAG ATTGAAGATT TCTGTTGAAA CTTACACTGT | 880 |
| | TTCATCTCAG CTTTTGATGG TACTGATGAG TCTTGATCTA | 920 |
| 35 | GATACAGGAC TGGTTCCTTC CTTAGTTTCA AAGTGTCTCA | 960 |

- 39 -

ATAGTGGAAA TACAGTAACG AGTTGGCCTA GCCTCGC

37

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

10 CCCAGCTGGG TCGGGCCTAA GCGCCGGGCC CGT

33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

20 GTGGCTCTTT AACAACTTT GCTTGTCCCG ATA

33

20 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAAGTGGTCT ATCCTGTACT TACCACAACA CCT

33

(2) INFORMATION FOR SEQ ID NO:7:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

35 TGTATACTCT GAAAGAGCGA TGCCTCCAGG T

31

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

| | | | | | | | | | | | | | | | | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| | Pro | Arg | Leu | Arg | Tyr | Asn | Ser | Leu | Arg | Cys | Trp | Arg | Ile | Leu | Leu | |
| | | | | | 5 | | | | | 10 | | | | | 15 | |
| | Arg | Thr | Arg | Thr | Ala | Ser | Gly | Arg | Leu | Phe | Pro | Arg | Ala | Arg | Ser | |
| 5 | | | | | 20 | | | | | 25 | | | | | 30 | |
| | Ile | Leu | Tyr | Arg | Ala | Arg | Ala | Lys | Thr | Thr | Glu | Val | Asp | Ser | Gly | |
| | | | | | 35 | | | | | 40 | | | | | 45 | |
| | Ala | Arg | Thr | Gln | Leu | Arg | Pro | Ala | Ser | Asp | Pro | Arg | Ile | Pro | Arg | |
| | | | | | 50 | | | | | 55 | | | | | 60 | |
| | Arg | Pro | Ala | Arg | Val | Val | Trp | Ile | Ala | Glu | Gly | Met | Pro | Arg | Arg | |
| | | | | | 65 | | | | | 70 | | | | | 75 | |
| | Ala | Glu | Asn | Trp | Asp | Glu | Ala | Glu | Val | Gly | Ala | Glu | Glu | Ala | Gly | |
| 10 | | | | | 80 | | | | | 85 | | | | | 90 | |
| | Val | Glu | Glu | Tyr | Gly | Pro | Glu | Glu | Asp | Gly | Gly | Glu | Glu | Ser | Gly | |
| | | | | | 95 | | | | | 100 | | | | | 105 | |
| | Ala | Glu | Glu | Ser | Gly | Pro | Glu | Glu | Ser | Gly | Pro | Glu | Glu | Leu | Gly | |
| | | | | | 110 | | | | | 115 | | | | | 120 | |
| | Ala | Glu | Glu | Glu | Met | Glu | Ala | Gly | Arg | Pro | Arg | Pro | Val | Leu | Arg | |
| | | | | | 125 | | | | | 130 | | | | | 135 | |
| 15 | Ser | Val | Asn | Ser | Arg | Glu | Pro | Ser | Gln | Val | Ile | Phe | Cys | Asn | Arg | |
| | | | | | 140 | | | | | 145 | | | | | 150 | |
| | Ser | Pro | Arg | Val | Val | Leu | Pro | Val | Trp | Leu | Asn | Phe | Asp | Gly | Glu | |
| | | | | | 155 | | | | | 160 | | | | | 165 | |
| | Pro | Gln | Pro | Tyr | Pro | Thr | Leu | Pro | Pro | Gly | Thr | Gly | Arg | Arg | Ile | |
| | | | | | 170 | | | | | 175 | | | | | 180 | |
| | His | Ser | Tyr | Arg | Gly | His | Leu | Trp | Leu | Phe | Arg | Asp | Ala | Gly | Thr | |
| | | | | | 185 | | | | | 190 | | | | | 195 | |
| 20 | His | Asp | Gly | Leu | Leu | Val | Asn | Gln | Thr | Glu | Leu | Phe | Val | Pro | Ser | |
| | | | | | 200 | | | | | 205 | | | | | 210 | |
| | Leu | Asn | Val | Asp | Gly | Gln | Pro | Ile | Phe | Ala | Asn | Ile | Thr | Leu | Pro | |
| | | | | | 215 | | | | | 220 | | | | | 225 | |
| | Val | Tyr | Thr | Leu | Lys | Glu | Arg | Cys | Leu | Gln | Val | Val | Arg | Ser | Leu | |
| | | | | | 230 | | | | | 235 | | | | | 240 | |
| | Val | Lys | Pro | Glu | Asn | Tyr | Arg | Arg | Leu | Asp | Ile | Val | Arg | Ser | Leu | |
| | | | | | 245 | | | | | 250 | | | | | 255 | |
| 25 | Tyr | Glu | Asp | Leu | Glu | Asp | His | Pro | Asn | Val | Gln | Lys | Asp | Leu | Glu | |
| | | | | | 260 | | | | | 265 | | | | | 270 | |
| | Arg | Leu | Thr | Gln | Glu | Arg | Ile | Ala | His | Gln | Arg | Met | Gly | Asp | | |
| | | | | | 275 | | | | | 280 | | | | | | |

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGTAGTGTC CTGTATTTAG TG

22

10 (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTTGGCTATG GGTAGAATTG G

21

(2) INFORMATION FOR SEQ ID NO:14:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

25 CAGGGTAGCC TTGATCTAAG T

21

(2) INFORMATION FOR SEQ ID NO:15:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

35 GGAGGTCCTG AGAATATGTG TCC

23

- 40 -

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TACCATCAAA AGCTGAGATG AACAGTGTA AGT

33

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acid residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Glu Glu Tyr Gly Pro Glu Glu Asp Gly Gly Glu Glu Ser Gly
5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acid residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Thr Gly Arg Arg Ile His Ser Tyr Arg Gly His Leu
5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACAAAGTGAT GCCTTGTAGC TG

22

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- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GATGGGCTTC TGGTTAACCA AACT

24

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(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TG TTCAGGCA CACAGTAGAT G

21

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CATCTTCTGC AATCGCAGTC CGCGCGT

27

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CAAAAGCTGA GATGAAACAG TGTAAGT

27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTTTG GTTAA CCAGAAGCCC ATCGT

25

(2) INFORMATION FOR SEQ ID NO:20:

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WHAT IS CLAIMED IS:

1. A phage containing a VHL disease gene having the identifying characteristics of ATCC deposit number ____.

5 2. A VHL disease gene sequence, or analogs thereof, having the sequence according to SEQ ID NO. 1.

3. A VHL disease gene encoding the protein, or analogs thereof, containing SEQ ID NO. 2.

10 4. A method for detecting carriers of the VHL disease gene comprising:
analyzing DNA of a subject for mutations of the VHL disease gene associated with VHL disease.

15 5. The method of claim 4, wherein said step of analyzing comprises Southern blot analysis.

20 6. The method of claim 5 wherein the probe used in said Southern blot analysis is derived from a wild-type VHL disease gene sequence.

7. The method of claim 6, wherein said sequence is a cDNA.

25 8. The method of claim 7, wherein said cDNA, or analogs thereof, has a sequence according to SEQ ID NO. 1.

30 9. The method of claim 4, wherein said step of analyzing is carried out by PCR-SSCP.

10. The method of claim 9, wherein the primers used in PCR-SSCP are derived from a cDNA, or analogs thereof, having a cDNA sequence according to SEQ ID NO. 1.

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11. The method of claim 10, wherein said primers have nucleic acid sequence according to SEQ ID NO. 3 through SEQ ID NO. 8.

5 12. A method for detecting carriers of the VHL disease gene comprising:
analyzing RNA of a subject for mutations or alterations in VHL-specific mRNA associated with VHL disease.

10 13. The method of claim 12, wherein said step of analyzing comprises RT-PCR.

15 14. The method of claim 13, wherein primers used in said RT-PCR are derived from a cDNA, or analogs thereof, having a cDNA sequence according to SEQ ID NO. 1.

20 15. The method of claim 14, wherein said primers have a sequence according to SEQ ID NO. 17 through SEQ ID NO. 20.

16. Primers derived from the VHL disease gene sequence.

25 17. The primers of claim 15, wherein said sequence is a cDNA.

30 18. The primers of claim 16, wherein said cDNA, or analogs thereof, has a sequence according to SEQ ID NO. 1.

35 19. The primers of claim 17, wherein said primers have nucleic acid sequences selected from the group consisting of SEQ ID NO. 3 through SEQ ID NO. 8, and SEQ ID NO. 17 through SEQ ID NO. 20.

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20. A diagnostic kit for use in detecting carriers of the VHL disease gene, said kit comprising:
primers having nucleic acid sequences selected from the group consisting of SEQ ID NO. 3 through
5 SEQ ID No. 8, and SEQ ID NO. 17 through SEQ ID NO. 20.

21. A method for detecting carriers of the VHL disease gene comprising:
analyzing the protein of a subject for
10 alterations in VHL protein expression associated with VHL disease.

22. The method of claim 20, wherein said step of analyzing comprises Western blotting.

15 23. The method of claim 21, wherein the antibody used in said Western blotting is directed against VHL protein.

20 24. A recombinant VHL protein derived from the gene sequence of claim 2.

25. An antibody to the VHL protein.

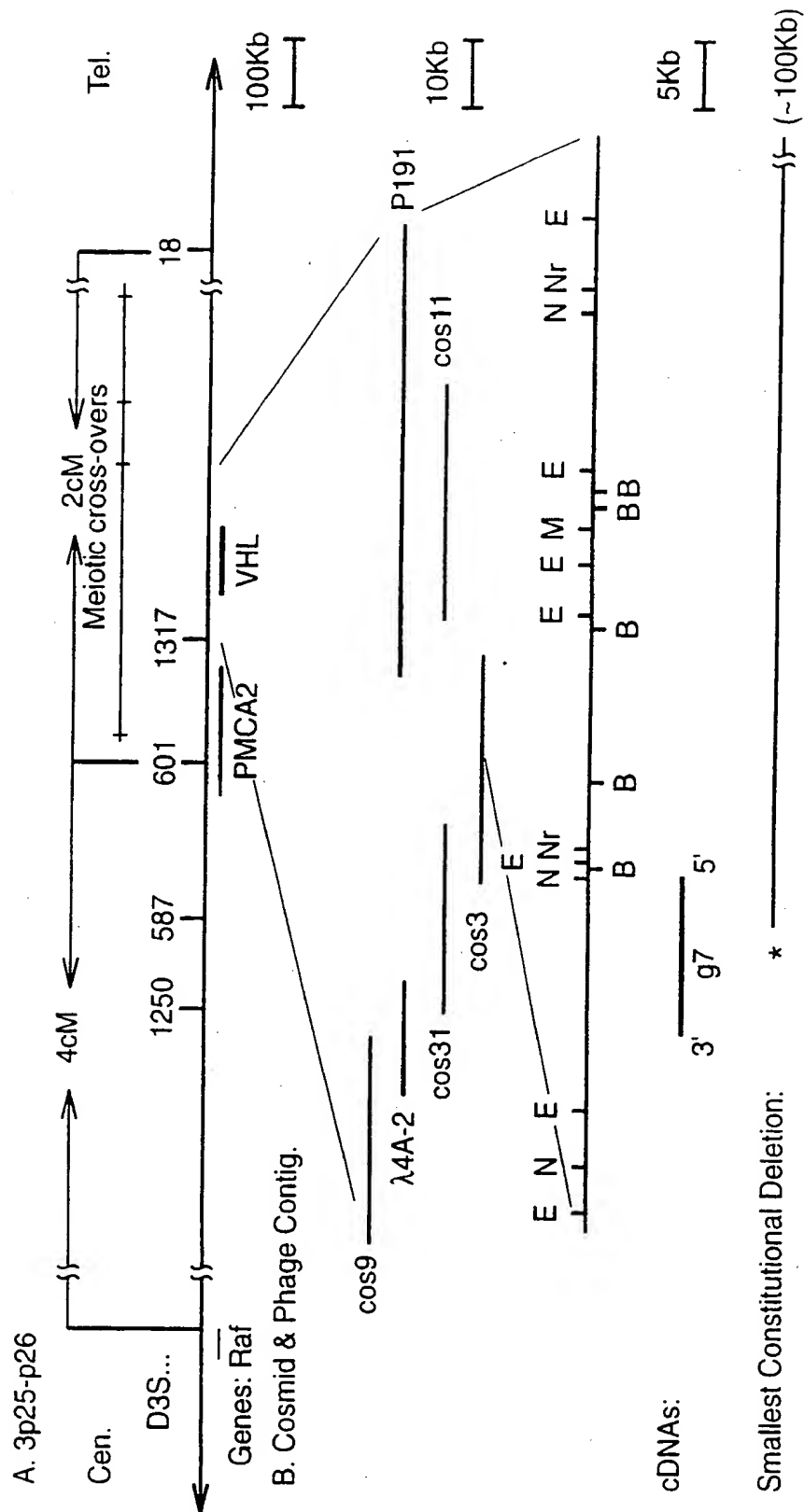
25 26. The antibody of claim 25, wherein said antibody is selected from the group consisting of polyclonal and monoclonal antibodies.

30

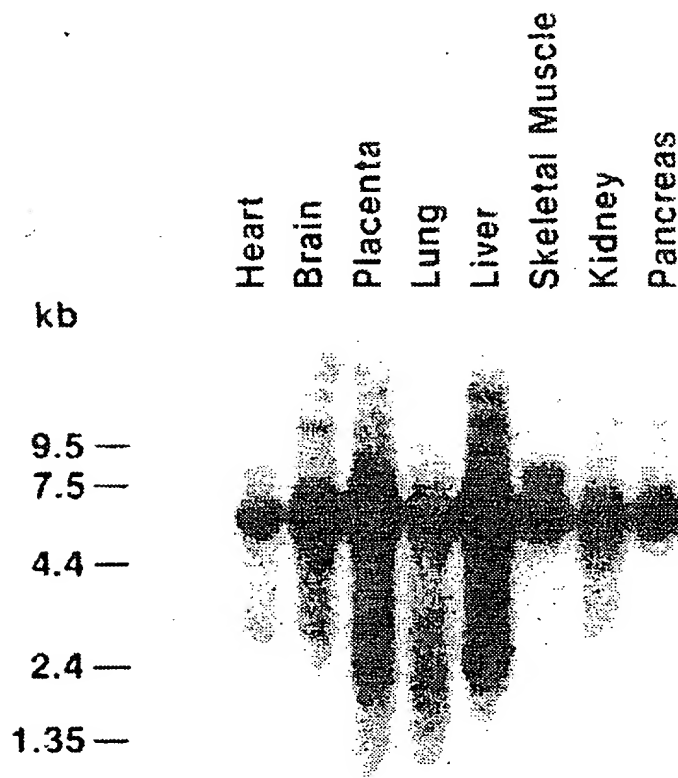
35

1/9

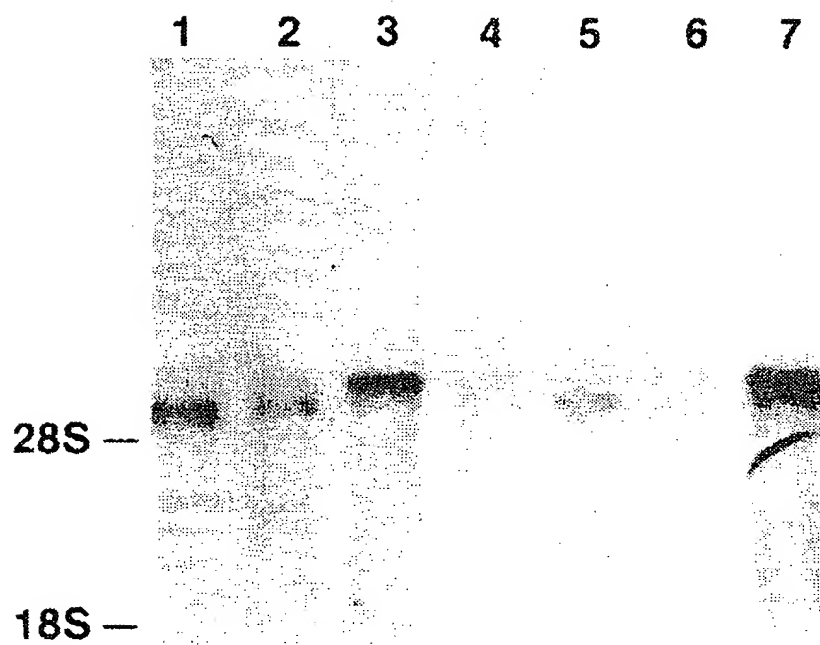
FIG. 1



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FIG. 2A

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FIG. 2B

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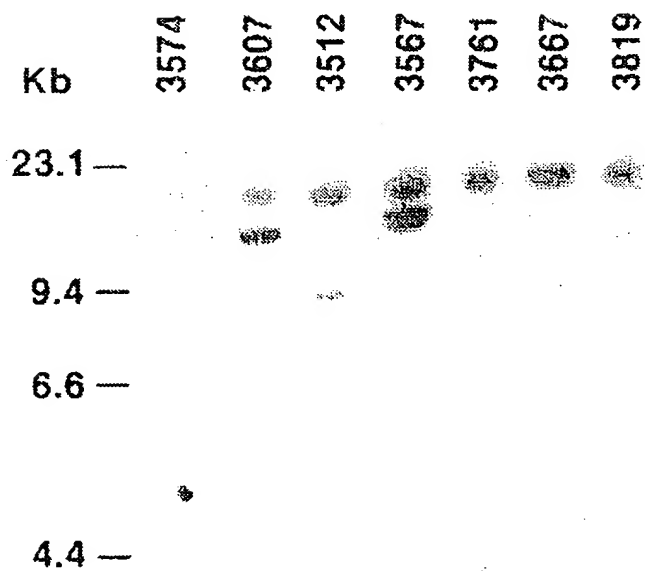
FIG. 3A

FIG. 3B

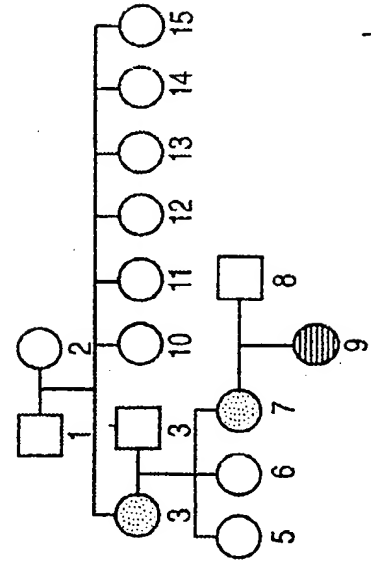
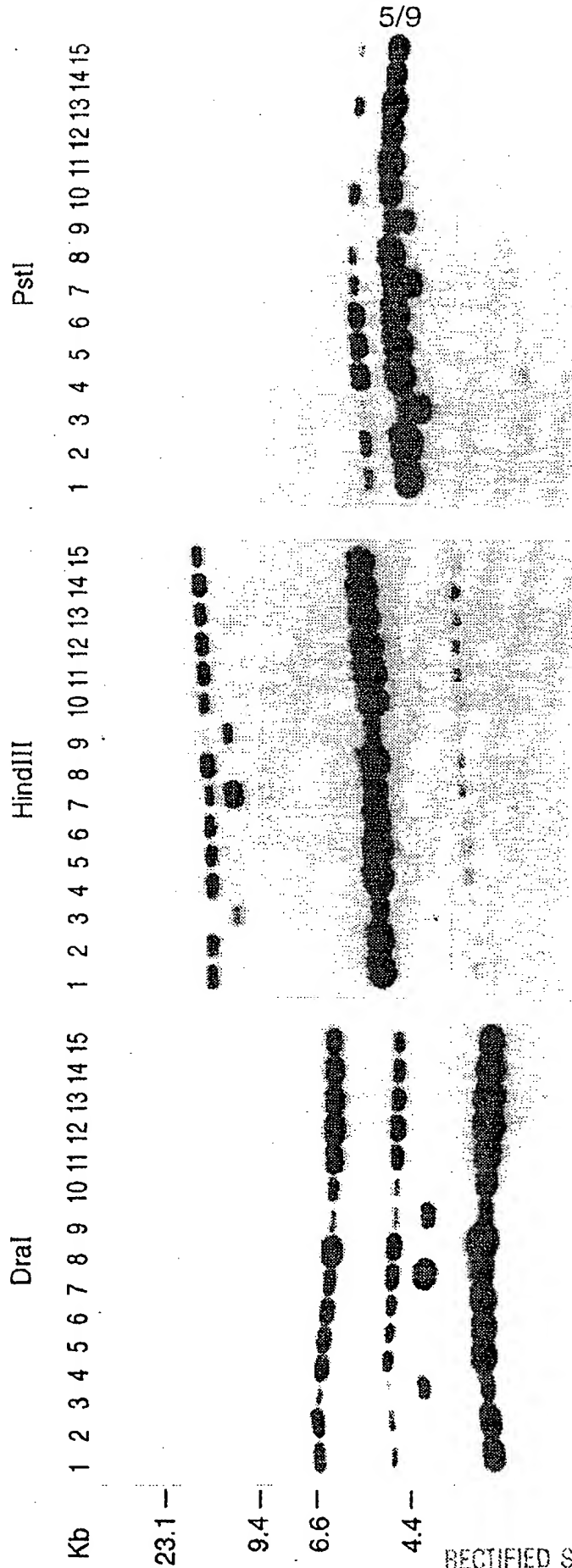
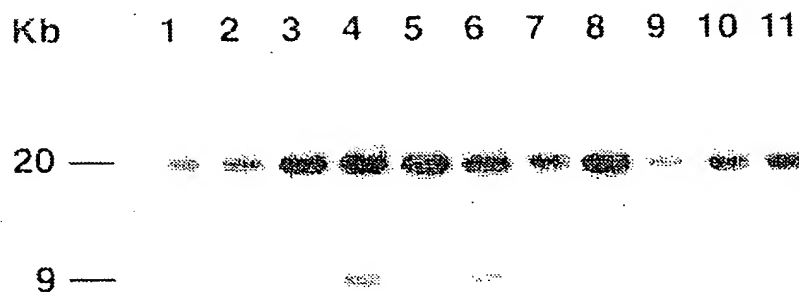
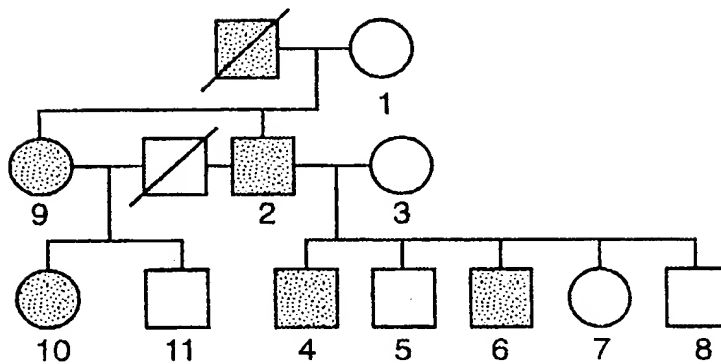


FIG. 3C

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FIG. 3D**FIG. 3E**

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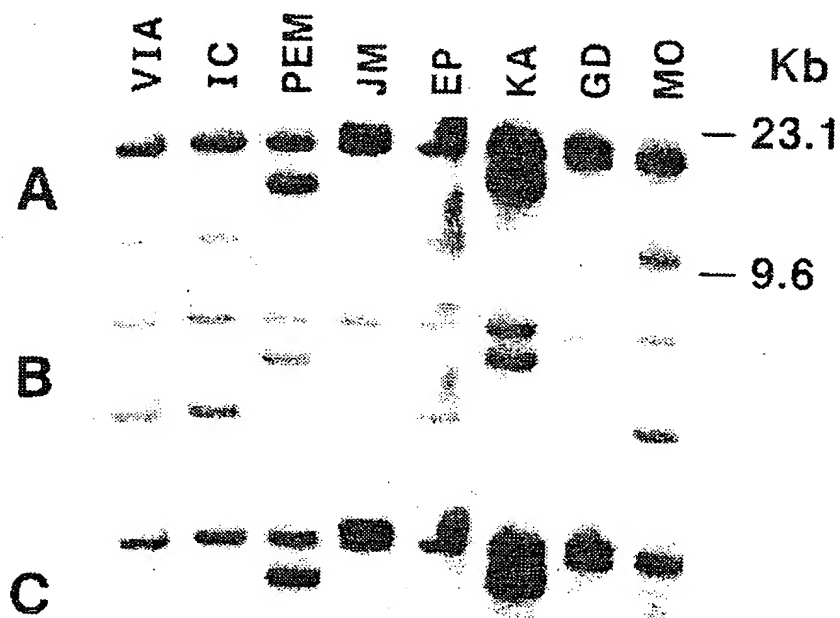
FIG. 4

FIG. 5B

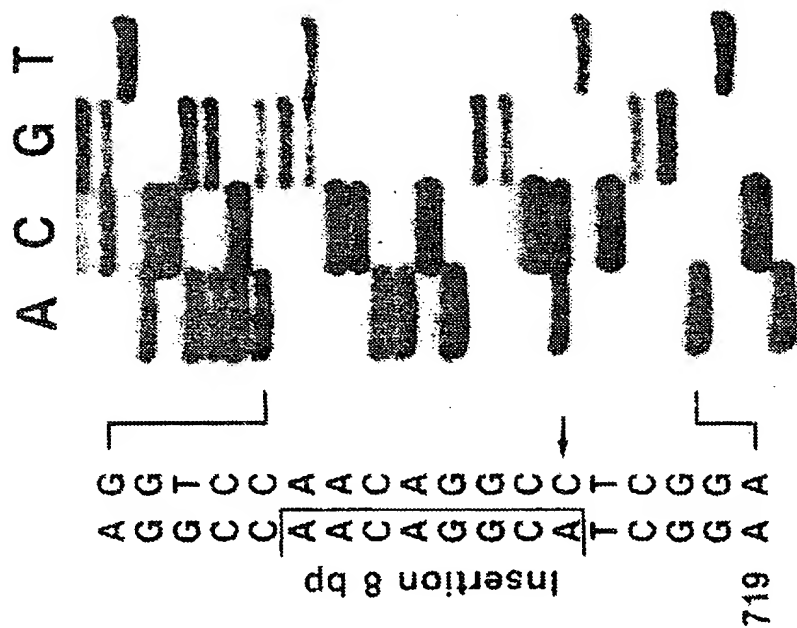
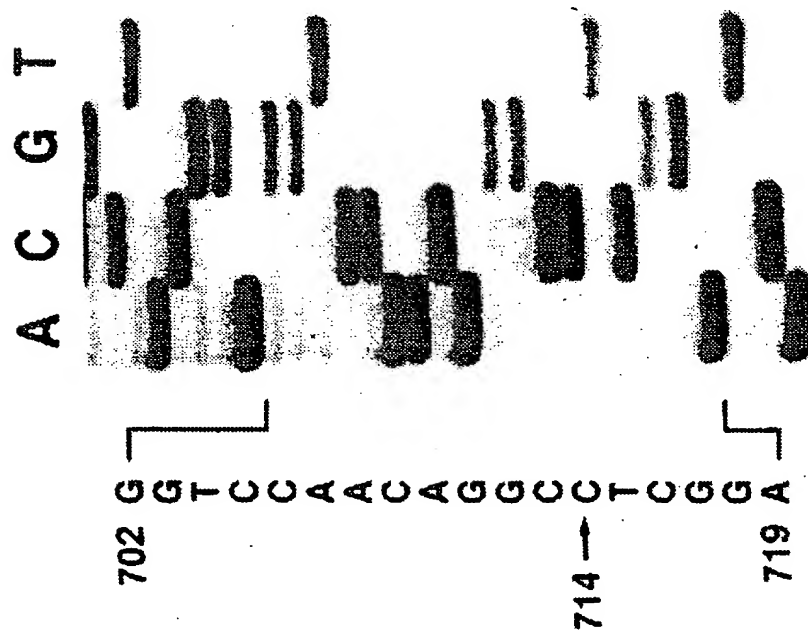
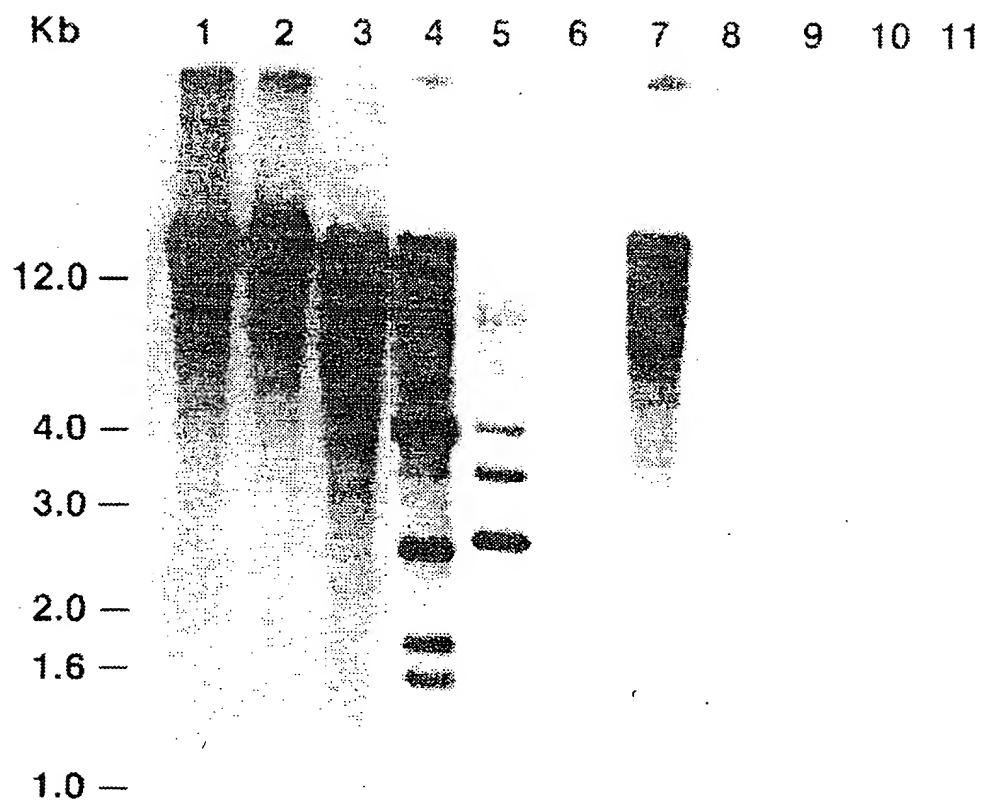


FIG. 5A



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FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/05378

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/12 C12Q1/68 C07K13/00 C07K15/28 G01N33/574
 G01N33/577 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C12Q C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| A | <p>PROC. NATL. ACAD SCI. vol. 88, no. 7, 1 April 1991, NATL. ACAD SCI., WASHINGTON, DC, US; pages 2864 - 2868 B.R. SEIZINGER ET AL. 'Genetic flanking markers refine diagnostic criteria and provide insights into the genetics of Von Hippel Lindau disease' cited in the application the whole document</p> <p style="text-align: center;">--- -/--</p> | 1-20 |

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

* Special categories of cited documents:

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- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

23 August 1994

Date of mailing of the international search report

31.08.94

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Fax (+31-70) 340-3016

Authorized officer

Hornig, H

International Application No.
PCT/US 94/05378

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|--------------------------|
| A | J. MED. GENETICS vol. 30, no. 2 , February 1993 , BMJ PUBL. GROUP, LONDON, UK; pages 104 - 107 F.M. RICHARDS ET AL. 'Detailed genetic mapping of the Von Hippel-Lindau disease tumour suppressor gene' cited in the application see page 105, right column, line 43 - page 107, left column, line 43 --- | 1-20 |
| A | CANCER RESEARCH vol. 53, no. 4 , 15 February 1993 , WARVERLY PRESS INC., BALTIMORE,US; pages 861 - 867 F. LATIF ET AL. 'Von Hippel-Lindau Syndrome: Cloning and Identification of the Plasma membrane Ca ⁺⁺ -transporting ATPase isoform 2 gene that resides in the Von Hippel-Lindau gene region' cited in the application the whole document --- | 1-3 |
| A | HUMAN MOLECULAR GENETICS vol. 2, no. 3 , March 1993 , OXFORD UNIVERSITY PRESS, CAMBRIDGE, UK; pages 279 - 282 P.A. CROSSEY ET AL. 'Genetic linkage between Von Hippel-Lindau disease and three microsatellite polymorphisms refines the localisation of the VHL locus' the whole document --- | 1-3 |
| O,P, X | CYTOGENET. CELL GENET. vol. 65 , 1994 page 41 M.L. LERMAN ET AL. 'Molecular cloning of the Von Hippel-Lindau tumor suppressor gene' Report of the fourth international workshop on human chromosome 3 mapping 1993; held on May 14-15, 1993 in Groningen, The Netherlands; see page 41, left column, paragraph 2 - right column, paragraph 1 --- | 1-3 |
| P,X | SCIENCE vol. 260 , 28 May 1993 , AAAS, WASHINGTON, DC, US; pages 1317 - 1320 F. LATIF ET AL. 'Identification of the von Hippel-Lindau disease tumor suppressor gene' see page 1317, middle column, line 1 - page 1320, left column, line 11 --- | 1-10, 12-14, 16,18 |
| | --- | |

INTERNATIONAL SEARCH REPORT

Internat. Application No.

PCT/US 94/05378

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|---|-----------------------|
| P,X | <p>CANCER REASERCH vol. 54, no. 9 , 1 May 1994 , WARVERLY PRESS INC., BALTIMORE,US; pages 2486 - 2491 I. KUZMIN ET AL. 'One-megabase yeast artificial chromosome and 400-kilobase cosmid-phage contigs containing the Von Hippel-Lindau tumor suppressor and Ca⁺⁺-transporting adenosine triphosphatase isoform 2 genes' see page 2487, left column, line 1 - page 2489, left column, line 37; figures 1,2 -----</p> | 1-10 |